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Species differences in courtship acoustic signals among five Lake Malawi cichlid species (*Pseudotropheus* spp.)

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Male courtship acoustic signals from five Lake Malawi cichlid fish species of the *Pseudotropheus zebra* complex were recorded and compared. Sounds made by males of *P. zebra*, *Pseudotropheus callainos* and the undescribed species known as *Pseudotropheus* 'zebra gold' from Nkhata Bay, and *Pseudotropheus emmiltos* and *Pseudotropheus faizilberi* from Mphanga Rocks, differed significantly in the number of pulses and in pulse period. The largest differences in acoustic variables were found among the sympatric Mphanga Rocks species that, in contrast to the other three species, show relatively minor differences in male colour and pattern. These findings suggest that interspecific mate recognition is mediated by multimodal signals and that the mass of different sensory channels varies among sympatric species groups. This study also showed that sound peak frequency was significantly negatively correlated with male size and that sound production rate increased significantly with courtship rate.

Key words: acoustic communication; courtship; mate choice; *P. zebra* complex; reproductive isolation; sound production.

INTRODUCTION

Cichlids from the African Great Lakes have undergone some of the fastest and most extensive adaptive radiations among vertebrates (Turner, 1999; Albertson *et al.*, 2003). From Lake Malawi alone, at least 450–600 endemic species have been recorded (Genner *et al.*, 2004). Most of these species are believed to have arisen within the lake catchment within a relatively short period of time, estimated at between 700 000 and 4 million years (Turner, 1999; Genner *et al.*, 2007). Many authors have proposed that sexual selection driven by female choice acting on male courtship colours may be a significant influence on the

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rapid speciation of these fishes (Dominey, 1984; McKaye, 1991; Genner & Turner, 2005). Visual cues were found to be relevant for interspecific mate recognition (Knight & Turner, 1999; Jordan *et al.*, 2003), but recent studies have pointed out that chemical (Plenderleith *et al.*, 2005) and acoustic (Amorim *et al.*, 2004) signals may also be important.

The recognition of species-specific acoustic signals can promote reproductive isolation and influence speciation processes in sympatric species (Ryan & Rand, 1993; Wells & Henry, 1998). Species recognition based on mating acoustic signals has been suggested for several teleosts (Crawford *et al.*, 1997; Amorim *et al.*, 2004) and was verified in damselfishes (Pomacentridae) (Myrberg *et al.*, 1978; Spanier, 1979). Males of several African cichlids are known to produce sounds during courtship (Lobel, 1998; Amorim *et al.*, 2004; Amorim, 2006). A preliminary study by Amorim *et al.* (2004) found statistically significant differences between some variables of the sounds produced in the early stage of courtship by males of three closely related species from Lake Malawi, *Pseudotropheus zebra* (Boulenger, 1899), *Pseudotropheus* 'zebra gold' (Ribbink *et al.*, 1983) and *Pseudotropheus callainos* Stauffer & Hert, 1992. If these differences are detected by females and influence mating decisions, acoustic communication may have an important role in the evolution of reproductive isolation and consequently on the impressive rate of speciation of these fishes.

In the present study, male courtship sounds of five *Pseudotropheus* species from Lake Malawi are compared: three sympatric species (*P. zebra*, *P.* 'zebra gold' and *P. callainos*) exhibit distinct colours and patterns, while the other two species [*Pseudotropheus emmiltos* (Stauffer, Bowers, Kellogg & McKaye, 1997) and *Pseudotropheus fainzilberi* Staeck, 1976] inhabiting another region in the lake are less divergent in their appearance. It is predicted that if species differences in courtship sounds are important in assortative mating, then acoustic signals will be more divergent among sympatric species that differ less in visual cues, such as in *P. emmiltos* and *P. fainzilberi*.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The study species belong to the *P. zebra* complex, one of the most-species rich mbuna cichlid groups endemic to Lake Malawi. These are also classed as members of the subgenus *Maylandia*, also known by the junior synonym *Metriaclima* (Stauffer *et al.*, 1997). Males of this species complex are similar in morphological traits but differ in their breeding colours (Fig. 1). *Pseudotropheus zebra*, *P. callainos* and the undescribed species *P.* 'zebra gold' co-occur in Nkhata Bay (Fig. 1), on the western shore of Lake Malawi (11°36′ N; 34°17′ E) in reproductive isolation (van Oppen *et al.*, 1998). *Pseudotropheus zebra* males are blue with black vertical bars, *P. callainos* males are blue without bars and *P.* 'zebra gold' males are yellow with brown vertical bars. At Mphanga Rocks (10°45′ S; 34°67′ E) off the north-western shore of the lake (Fig. 1), *P. emmiltos* and *P. fainzilberi* co-occur sympatrically. Males of both species are blue with dark vertical bars, but differ in smaller details of their breeding colours: *P. emmiltos* males have bright orange-red dorsal fins, while *P. fainzilberi* have blue dorsal fins with prominent black horizontal bands.

Populations of the two sympatric groups do not differ in the degree of sympatry or relatedness. All three populations at Nkhata Bay are fully sympatric, as are both at

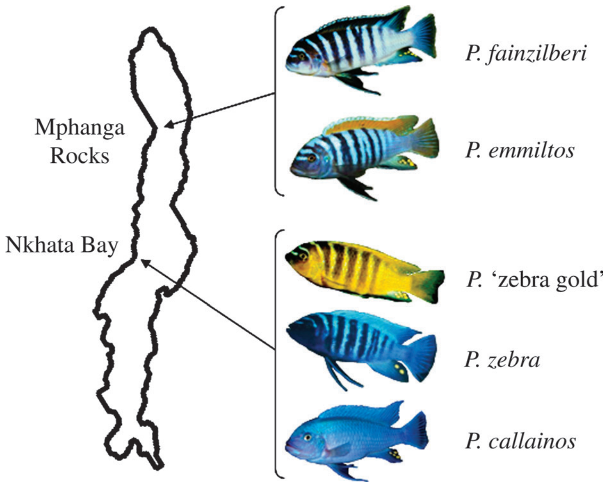

FIG. 1. Comparison of interspecific differences of male courtship acoustic signals was carried out using five species from the northern-west part of Lake Malawi. *Pseudotropheus fainzilberi* and *Pseudotropheus emmiltos* are native to Mphanga Rocks and males are blue with black vertical bars and differ mainly in the colour of the dorsal fin. *Pseudotropheus* 'zebra gold', *Pseudotropheus zebra* and *Pseudotropheus callainos* are found at Nkhata Bay. Males from the last three species differ more extensively in their courtship colour, with *P.* 'zebra gold' exhibiting brown vertical bars on a yellow background, *P. zebra* black vertical bars on a blue background, while *P. callainos* are plain blue.

Mphanga Rocks. Although the study species at Nkhata Bay show microhabitat preferences, including some depth preference, substantial overlap in breeding ranges of all three species have been demonstrated (van Oppen *et al.*, 1998). Similarly, at Mphanga Rocks, the depth range of *P. fainzilberi* was fully contained within that of *P. emmiltos*, although individuals of the latter species were found at greater depths than those of the former (pers. obs.). Phylogenetic relationships of the study taxa have not been fully resolved. Mitochondrial DNA studies of mbuna populations have typically shown poor phylogenetic resolution, with extensive sharing of polymorphisms even between members of different genera. Genome-wide surveys of DNA polymorphisms (AFLPs) have shown that all of the *Pseudotropheus* (Maylandia) complex populations from the northern half of the lake (like the five taxa used in the present study) are very closely related (Allender *et al.*, 2003).

Males defend territories to which they try to attract females to spawn with by means of a series of stereotyped visual displays (Baerends & Baerends van Roon, 1950), acoustic signals (Amorim *et al.*, 2004) and chemical cues (Plenderleith *et al.*, 2005). During spawning, females take both eggs and sperm into their mouth, where the eggs are fertilized, and leave the male territory to mouthbrood in quiet shelters for up to 3 weeks. Each group of the studied sympatric species mate assortatively in the laboratory (Knight *et al.*, 1998; Plenderleith *et al.*, 2005), indicating that reproductive isolation can be maintained by direct mate choice alone.

All fishes used were first generation laboratory stock (*c.* 200 adults per species), bred from parents collected at Nkhata Bay and Mphanga Rocks. Fishes were kept in 220 l tanks in a 5500 l re-circulation system, with a density of *c.* 40 fishes per tank, under a 12L:12D regime and fed on a mixture of commercial fish flakes and pellets. Water temperature was kept at 25–27° C.

SOUND RECORDING AND ANALYSIS

Pseudotropheus spp. males were recorded in experimental tanks (Nkhata Bay species: two tanks 1200 × 600 × 450 mm high; Mphanga Rocks: four tanks (1000 × 500 × 400 mm high) divided into three compartments by two opaque removable partitions. Lateral compartments (300 mm wide) held a single male, with a terracotta pot that served as refuge and as a prospective spawning site, and the central compartment (*c.* 400 mm wide) housed five to seven females throughout the experiment. Each tank housed a single species. Recording tanks were placed on top of thick layers of rock–wool that insulated tanks from external noise transmitted through floor vibrations. Males were left to acclimatize for a minimum of 12 h before the start of recording trials.

Approximately 5–10 min prior to the start of a recording session all electric appliances (aeration, filters and lights) were switched off. Each recording session started when one of the opaque partitions was removed, allowing the focal male free access to females for 20 min, after which males were placed back in their lateral compartment. Each male was recorded in a maximum of three sessions. Once recordings were complete, the tested subject was weighed (mass, M), measured (standard length, L_S), returned to a stock tank and replaced with another male of the same species. Males were identified by electronic tags that were previously inserted in their abdominal cavity or by natural marks such as number of egg spots in the anal fin.

Sounds were recorded using two High Tech 94 SSQ hydrophones (High Tech Inc., Gulfport, MI, U.S.A.) (sensitivity of 165 dB re $1\mathrm{V}\mu\mathrm{Pa}^{-1}$) to improve the probability of recording sounds close to the sound emitter, and a Pioneer DVD Recorder DVR-3100 (Tokyo, Japan) (sampling frequency 48 kHz, 24 bit resolution). This audio chain had a flat frequency response up to 6 kHz ± 1·5 dB. Sounds were analysed with Adobe Audition 2.0 (Adobe Systems, Inc.) and Raven 1.2.1 for Windows (Cornell Lab of Ornithology). Acoustic analysis only considered sounds associated with the behaviour quiver that is characteristic of the early stages of courtship (Baerends & Baerends van Roon, 1950). Moreover, only sounds that showed a clear structure, typically registered at a distance of 1–2 total lengths of the focal fish, were analysed. Recorded sounds could be attributed to the subject males because their intensity varied with distance from the hydrophone and were consistently associated with particular courtship displays, such as quiver and circle (Amorim *et al.*, 2004).

The following acoustic variables were analysed (Fig. 2; Amorim *et al.*, 2004): sound duration (ms); number of pulses in a sound; mean pulse period (average peak-to-peak interval between consecutive pulses, ms). In addition, two frequency peaks at *c.* 150 Hz (PF1) and at 450 Hz region (PF2) were measured (Fig. 2). Temporal features were measured from oscillograms and peak frequencies from power spectra based on 2048 point FFT with a Hamming window applied.

A total of 12 *P.* 'zebra gold', 12 *P. zebra*, 13 *P. callainos*, 13 *P. fainzilberi* and 14 *P. emmiltos* adult males were recorded and analysed (Table I provides details on male size and number of sounds recorded per male).

BEHAVIOUR RECORDING AND ANALYSIS

During sound recording sessions male courtship behaviour was also tallied. Male courtship behaviour includes the behavioural patterns quiver, dart, lead-swim and circle (Baerends & Baerends van Roon, 1950; Amorim *et al.*, 2004). Quiver rate (number min^{-1}), courtship rate (total number of courtship activities min^{-1}) and sound production rate (number min^{-1}) were considered for each recording session.

DATA ANALYSIS

One-way ANOVA was used to compare differences among species in the duration, number of pulses and pulse period of 'quiver' sounds. The square root transformation was applied to the number of pulses to meet the ANOVA assumptions of normality and homoscedasticity. Comparison among species for variables other than sound frequency were not controlled for the effect of L_S because the variables were not significantly correlated with L_S (Spearman rank correlation, r_s, $P > 0{\cdot}05$; Amorim *et al.*,

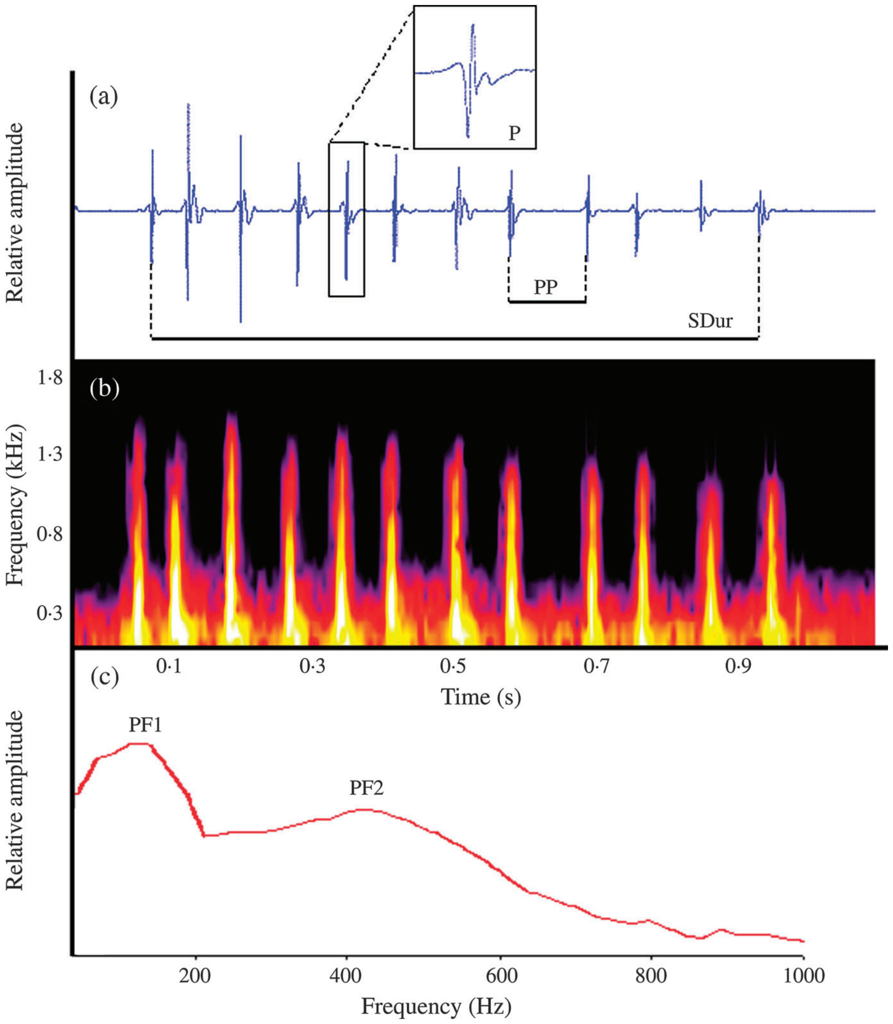

FIG. 2. (a) Oscillogram, (b) sonogram and (c) power spectrum of a typical courtship sound emitted by males of *Pseudotropheus* spp. (*P. fainzilberi* in the example) during 'quivering'. Some of the acoustic variables measured are depicted in the figure: number of pulses (P is an example of a pulse), sound duration (SDur), pulse period (PP), and peak frequency 1 (PF1) and 2 (PF2). Relative amplitude is shown in the *y*-axis of the oscillogram and the power spectrum.

2004). Because the dominant sound frequency is dependent on male size (Amorim *et al.*, 2004), however, an ANCOVA was conducted to test differences among species for the frequency variables PF1 and PF2, using L_S as a covariate. The assumption of slope parallelism was tested before carrying out the above ANCOVA models (PF1 and PF2, both d.f. = 4, 54, $P > 0{\cdot}05$).

Spearman rank correlation, r_s, was used to test whether quiver rate and courtship rate were related to sound production rate in each species. In addition, sound production rate was compared among species with ANOVA. Sound production rate was $\log_{10}(x + 1)$ transformed to meet the ANOVA assumptions. An average of two recording sessions were considered per male. All statistical analyses were conducted using Statistica 7.1 for Windows (StatSoft, Inc.).

TABLE I. Male *Pseudotropheus* spp. standard length (L_S), mass (M) and number of analysed sounds. Values are mean (range)

	P. 'zebra gold' ($n = 12$)	*P. zebra* ($n = 12$)	*P. callainos* ($n = 13$)	*P. fainzilberi* ($n = 13$)	*P. emmiltos* ($n = 14$)
L_S (mm)	107·3 (89·0–123·0)	107·7 (88·0–122·0)	97·7 (86·8–115·0)	118·7 (109·0–128·0)	126·3 (116·0–139·0)
M (g)	38·8 (23·5–56·1)	40·5 (22·0–57·7)	28·7 (20·7–46·3)	53·9 (41·5–77·3)	65·3 (54·9–77·9)
Number of sounds	16·7 (4–33)	16·5 (4–40)	18·4 (14–27)	11·0 (4–23)	9·7 (4–17)

RESULTS

Sound production by females was never observed during courtship interactions in any species. Males courted females mostly by repeated sequences of darting and quivering. During quivering, males from the five studied species commonly produced low-frequency pulsed sounds (Table II and Fig. 3). Quivering behaviour could last longer than sound production especially after the male emitted a few sounds.

When comparing all five *Pseudotropheus* species, no significant differences in sound duration were found (ANOVA, d.f. = 4, 59, $P > 0{\cdot}05$; Fig. 4). Sounds lasted *c.* 700 ms in all species. The number of pulses differed significantly among species (ANOVA, d.f. = 4, 59, $P < 0{\cdot}001$), with *P. emmiltos* producing the greatest number of pulses per sound (mean = 16·3 pulses), followed by *P.* 'zebra gold' (mean = 12·7) and by the three other species (means = 8·6–9·5; Fig. 4). Pulse period also showed significant interspecific differences (ANOVA, d.f. = 4, 59, $P < 0{\cdot}001$) with *P. emmiltos* producing pulses with significantly shorter periods, *i.e.* at a faster rate than the remaining species, followed by *P.* 'zebra gold' and *P. callainos*, and then by *P. zebra* and *P. fainzilberi* (Fig. 4). *Pseudotropheus* 'zebra gold' and *P. callainos* did not differ significantly in pulse period, nor did *P. zebra* and *P. fainzilberi* (Fig. 4). Mean pulse period was *c.* 50 ms for *P. emmiltos*, 70 ms for *P.* 'zebra gold' and *P. callainos*, and near 90 ms for the remaining species. Neither PF1 (ANCOVA, d.f. = 4, 58, $P > 0{\cdot}05$) nor PF2 (ANCOVA, d.f. = 4, 58, $P > 0{\cdot}05$) differed among species after controlling for the effect of male size (Fig. 4). Male L_S decreased significantly PF1 (ANCOVA, covariate L_S, d.f. = 4, 58, $P < 0{\cdot}05$) and especially PF2 (ANCOVA: covariate L_S, d.f. = 4, 58, $P < 0{\cdot}001$).

When comparing the two groups of sympatric species, the largest differences in acoustic variables were found among the Mphanga Rocks species both for number of pulses and pulse period. The largest mean pair-wise differences in the acoustic variables of the Nkhata bay group were found between *P.* 'zebra gold' and *P. zebra* that typically differed by 4 pulses and 21 ms in pulse period. In contrast, mean differences between Mphanga Rocks species were almost double than the previous and amounted to 7 pulses and 38 ms in pulse period.

Sound production rate was positively correlated with both quiver rate (n = 12–29, $P < 0{\cdot}001$) and total courtship rate (n = 12–29, $P < 0{\cdot}001$) in all five studied species. Sound production rate was significantly higher in *P. emmiltos* than in the other four species (ANOVA, d.f. = 4, 45, $P < 0{\cdot}001$), with *P. emmiltos* producing on average 1·4 sounds min^{-1} and the remaining species 0·7–0·8 sounds min^{-1}.

DISCUSSION

The present study demonstrated the existence of interspecific differences in the courtship sounds of members of the *P. zebra* complex that may allow species recognition. Males from the five species studied, including *P. fainzilberi* and *P. emmiltos* which were studied for the first time, produced low frequency pulsed sounds that differed in the number and rate of pulse production. These sounds were produced mostly when males were quivering to females.

TABLE II. Description of quiver-sound acoustic variables as overall mean (range), produced by males of *Pseudotropheus* spp. Overall means and ranges are based on individual fish means

Variables	*P.* 'zebra gold' ($n = 12$)	*P. zebra* ($n = 12$)	*P. callainos* ($n = 13$)	*P. fainzilberi* ($n = 13$)	*P. emmiltos* ($n = 14$)
Duration (ms)	774·1 (558·4–1022·4)	671·7 (421·4–856·8)	617·7 (349·4–1032·7)	722·6 (397·8–1117·6)	759·5 (301·8–987·4)
Number of pulses	12·7 (7·9–19·9)	8·6 (6·6–12·4)	9·5 (6·4–14·7)	9·0 (6·7–11·4)	16·3 (9·8–22·4)
Mean pulse period (ms)	65·9 (52·7–78·2)	86·8 (67·5–113·3)	72·5 (60·6–83·4)	86·7 (63·5–108·0)	48·4 (31·4–61·6)
PF1 (Hz)	151·8 (134·8–201·9)	155·6 (129·4–220·7)	149·7 (132·4–182·3)	138·0 (124·3–151·4)	133·7 (112·0–145·4)
PF2 (Hz)	476·9 (432·1–544·9)	488·8 (423·9–557·8)	519·5 (474·4–566·8)	473·2 (437·8–548·6)	448·4 (417·7–485·1)

PF1, peak frequency 1; PF2, peak frequency 2.

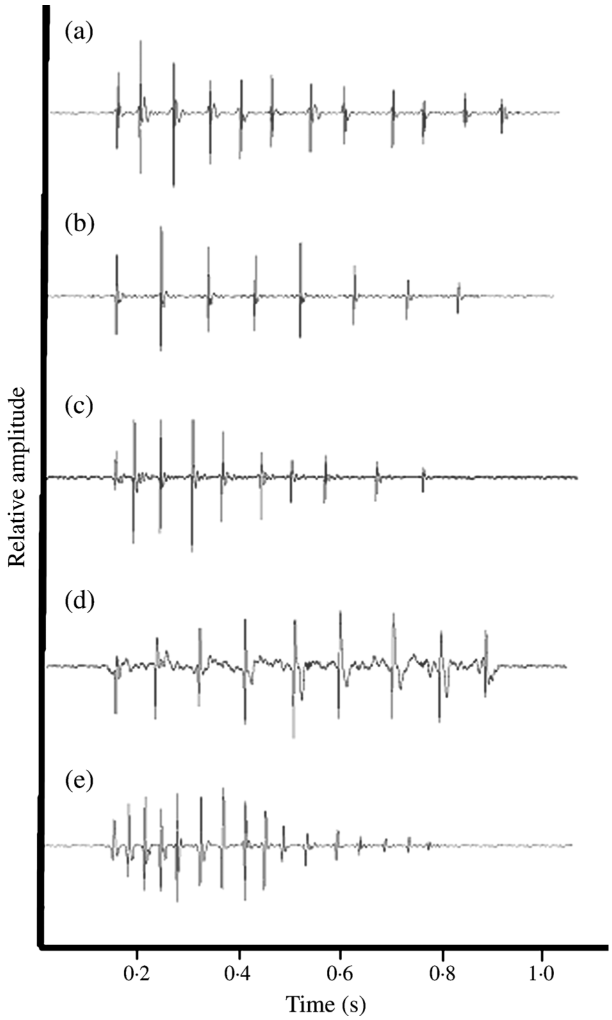

FIG. 3. Oscillograms of typical courtship sounds emitted by males of (a) *Pseudotropheus* 'zebra gold', (b) *Pseudotropheus zebra*, (c) *Pseudotropheus callainos*, (d) *Pseudotropheus fainzilberi* and (e) *Pseudotropheus emmiltos*. Differences among species and especially in *P. fainzilberi* and *P. emmiltos* can be observed in number of pulses and in the pulse period. Sound amplitude (relative amplitude) in oscillograms is not absolute and comparisons of this variable can only be made among pulses of the same sound.

The Nkhata Bay species differed in number of pulses and pulse period, with *P.* 'zebra gold' emitting longer sounds and with a higher number of pulses than *P. zebra* and *P. callainos*, and *P. zebra* exhibiting lower pulse rates (*i.e.* longer pulse periods). The present findings are consistent with the earlier study by Amorim *et al.* (2004) who found that *P.* 'zebra gold' males produced sounds with more pulses than *P. callainos* did.

According to the *a priori* hypothesis, *P. fainzilberi* and *P. emmiltos* from Mphanga Rocks presented larger differences in pulse number and period than the Nkhata Bay species, which showed greater differences in male colour.

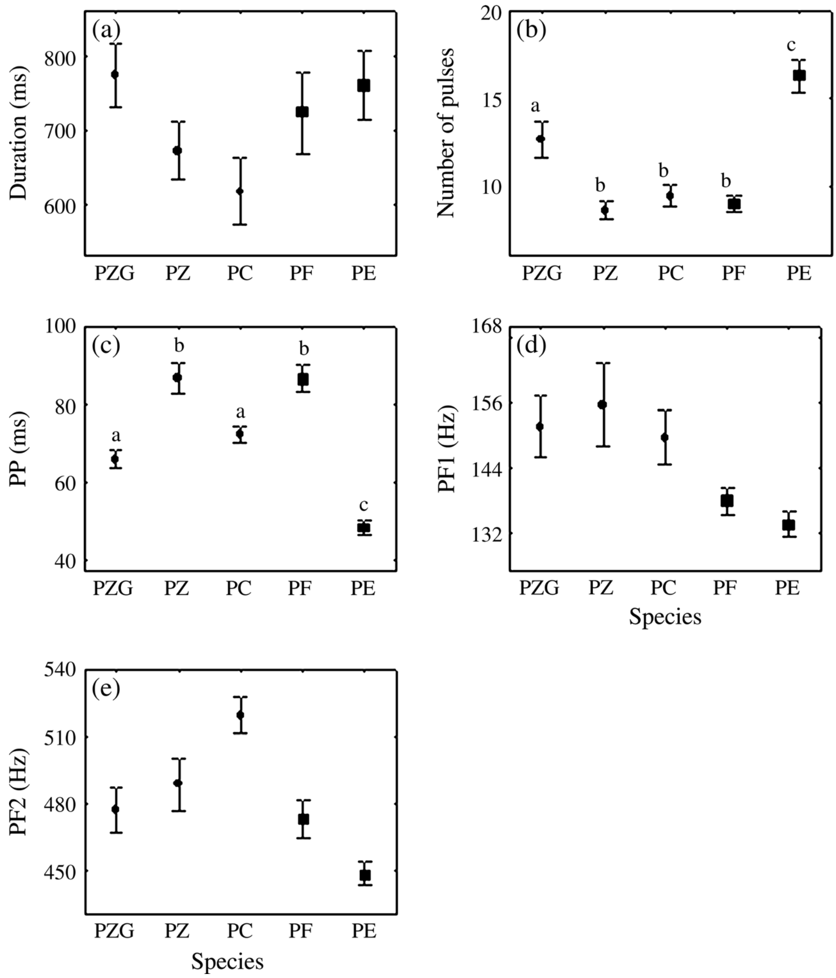

FIG. 4. Comparison of courtship mean ± S.E. sound variables: (a) duration, (b) number of pulses, (c) pulse period (PP), (d) peak frequency 1 (PF1) and (e) peak frequency 2 (PF2) among the five species: *Pseudotropheus* 'zebra gold' (PZG), *Pseudotropheus zebra* (PZ), *Pseudotropheus callainos* (PC), *Pseudotropheus fainzilberi* (PF) and *Pseudotropheus emmiltos* (PE) (●, Nkhata Bay species; ■, Mphanga Rock species). Sample sizes were n (PZG) = n (PZ) = 12; n (PC) = n (PF) = 13; n (PE) = 14. Significant differences for pair-wise comparisons (Tukey test, $P < 0{\cdot}05$) are indicated by different lower case letters. Notice that the largest differences in acoustic variables within bays were found among the Mphanga Rocks species.

Similarly, Nelissen (1978) has found that the number of colour patterns and sound types was inversely proportional in six Tanganyikan cichlid fishes.

In *Pseudotropheus* spp., as well as in other fishes, mate recognition and evaluation may involve the integration of different sensory components (Candolin, 2003). Acoustic signals of teleosts are thought to be part of a multimodal signal system as they are usually produced in tight association with particular visual displays (Amorim *et al.*, 2004) and because playback of sound alone often fails to elicit a response unless the sounds are accompanied by visual stimuli (Ladich, 2004). This study suggests that the acoustic sensory channel may have more weight in the multimodal courtship displays of the Mphanga Rocks species than in the Nkhata Bay ones. Consistent with this suggestion, *P. fainzilberi* and *P. emmiltos* failed to mate assortatively in laboratory mating trials when females had access only to visual signals from males (Plenderleith *et al.*, 2005).

Reports of sound production in other cichlid species indicate that they produce only one type of sound during courtship (Amorim, 2006), typically associated with the quivering behaviour, an early courtship behaviour when mate recognition is likely to occur (Amorim *et al.*, 2004; Ripley & Lobel, 2005). Other African cichlid species that are often closely related and sympatric also differ in the pulse number, pulse rate and in sound duration (Lobel, 2001; Rice & Lobel, 2004). These consistent differences in temporal patterning in cichlid courtship sounds suggest that these signal variables may play a role in interspecific recognition in sympatric species of cichlids, including members of the *P. zebra* complex. In fishes as in other taxa, temporal information is important for interspecific and intraspecific communication, such as species recognition (Winn, 1964; Honda-Sumi, 2005). In other fishes, *e.g.* Pomacentridae, several species of the genus *Stegastes* are sympatric and male courtship chirps show species-specific duration, number of pulses and pulse repetition rate (Myrberg *et al.*, 1978; Lobel & Mann, 1995). Playback experiments have confirmed that the number of pulses and pulse rate can promote species-specific recognition (Myrberg *et al.*, 1978; Spanier, 1979), demonstrating that species recognition based on acoustic cues occurs in fishes. These experiments, however, did not demonstrate that male chirps were effective in species isolation, as only the male response to playbacks was measured.

Premating mechanisms among sympatric species are essential for the maintenance of reproductive isolation in recently evolved species where hybridization is still possible. Divergent mating signals can be effective mechanisms in preventing hybridization (Qvarnström *et al.*, 2006) and closely related species may use different sensory channels to recognize conspecific mates (Rafferty & Boughman, 2006). Recent studies involving members of the *P. zebra* complex have emphasized the possible use of different sensory channels in the maintenance of reproductive isolation among sympatric species and allopatric forms. Choice experiments where olfactory and possibly also acoustic communication were prevented suggested that male colour, shape and pattern are the most important cues for mate recognition in some species (Jordan *et al.*, 2003; Kidd *et al.*, 2006). In other *P. zebra* complex species, however, olfactory cues must be present for preference for conspecific males to occur (Plenderleith *et al.*, 2005). Up to present there is no study that investigated the contribution of the acoustic channel for mate recognition by species of this rich group of Lake Malawi cichlids. Nevertheless, in view of the diversity of acoustic signals it is possible that different species may use this sensory channel balanced with the visual and olfactory ones in interspecific and intraspecific recognition and mate choice.

Notably, other sound features did not differ among the *Pseudotropheus* species but presented intraspecific variation associated with male traits, suggesting that some sound variables of signalling males might contain information about species identity while others could be used in the evaluation of conspecific males and hence in intraspecific mate choice. Sound peak frequency, in particular PF2, was negatively correlated with fish size in all studied species with PF2 decreasing on average by *c.* 2·2 Hz per mm increase in male L_S. This inverse relation between dominant frequency and fish size is common in cichlids (Rowland, 1978; Amorim *et al.*, 2003) and in other fishes (Myrberg *et al.*, 1993), and may be used by females as a cue in mate choice. For example, female damselfishes (Pomacentridae) prefer sounds of lower frequency that, as in *Pseudotropheus* spp. males, indicate a larger male body size (Myrberg *et al.*, 1986). The rate of sound production, that was positively correlated with quiver frequency and total courtship frequency in all five *Pseudotropheus* species, could also be used by females for assessing the condition and motivation of a conspecific male. Females of several taxa commonly have a preference for males that show a higher courtship display activity, as it may be related to higher genetic quality or other preferred male traits (Svensson *et al.*, 2004).

The present results suggest that different features of the courtship calls may contain information about species identity and intraspecific differences in traits, such as size, relevant to mate choice. In particular, the number of pulses and pulse period of courtship sounds are species-specific and these acoustic cues, in conjunction with visual and chemical information, may promote reproductive isolation. Finally, the present findings suggest that the weight of different sensory channels used in interspecific mate recognition may vary among sympatric species assemblages.

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